

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PAGE BLANK (USPTO)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

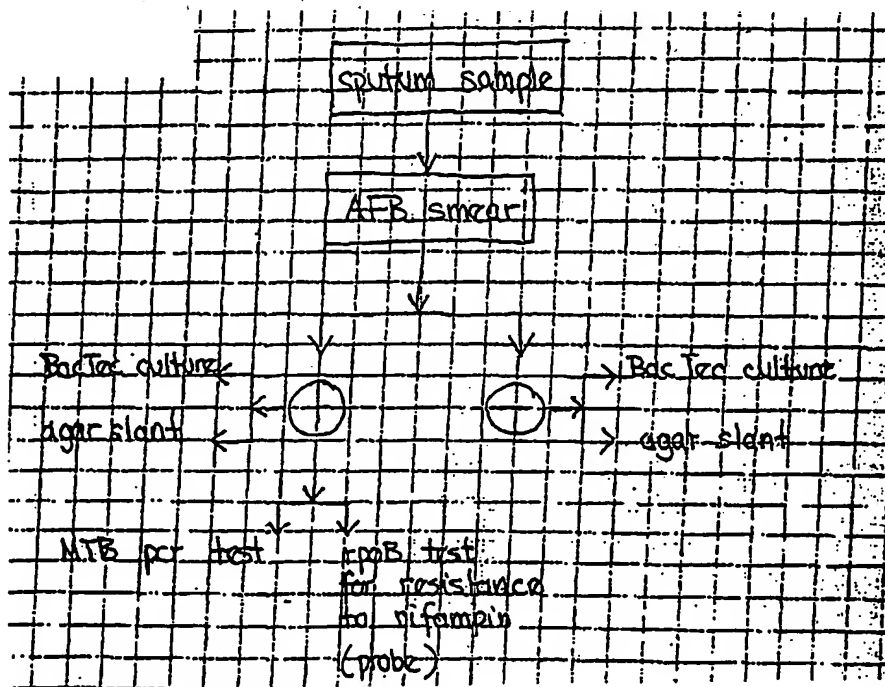
(51) International Patent Classification ⁷ : C12Q 1/68	A1	(11) International Publication Number: WO 00/36142 (43) International Publication Date: 22 June 2000 (22.06.00)
(21) International Application Number: PCT/CA99/01177 (22) International Filing Date: 10 December 1999 (10.12.99) (30) Priority Data: 60/111,794 11 December 1998 (11.12.98) US (71) Applicant (for all designated States except US): VISIBLE GENETICS INC. [CA/CA]; 700 Bay Street, P.O. Box 333, Toronto, Ontario M5G 1Z6 (CA). (72) Inventor; and (75) Inventor/Applicant (for US only): SHIPMAN, Robert [CA/CA]; #2-6240 Montevideo Road, Mississauga, Ontario L5N 2N7 (CA). (74) Agent: DEETH WILLIAMS WALL; National Bank Building, Suite 400, 150 York Street, Toronto, Ontario M5H 3S5 (CA).	(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: **METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN MYCOBACTERIUM TUBERCULOSIS**

(57) Abstract

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the *rpoB* (rifampin), *katG* (isoniazid), *oxyR-ahpC* PR (isoniazid), *mabA* (isoniazid), *rpsL/s12* (streptomycin), *16S/rrs* (streptomycin), *embB* (ethambutol), *pncA* (pyrazinamide), *gyrA* (ciprofloxacin) and *23S* (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing *M. tuberculosis*, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the

rpoB, *katG*, *rpsL/s12* and *23S* genes for the presence of antibiotic-resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

**METHOD AND KIT FOR THE CHARACTERIZATION OF
ANTIBIOTIC-RESISTANCE MUTATIONS IN
*MYCOBACTERIUM TUBERCULOSIS***

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing its uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of *M. tuberculosis* and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR-based or probe-based. These tests are used

- 2 -

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. Tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533 ^a
2.	Isoniazid	katG gene	codon 275/315/328 ^b
3.	Isoniazid	mabA gene	unknown ^c
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A ^e
6.	Pyrazinamide	pncA gene	codon 47/85 ^f
7.	Ethambutol	embB gene	codon 306 ^g
8.	Streptomycin	rpsL/s12 gene	codon 43/88 ^h
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95 ^j

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in its present form.

- 3 -

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the *rpoB* (rifampin), *katG* (isoniazid), *oxyR-ahpC PR* (isoniazid), *mabA* (isoniazid), *rpsL/s12* (streptomycin), *16S/rrs* (streptomycin), *embB* (ethambutol), *pncA* (pyrazinamide), *gyrA* (ciprofloxacin) and *23S* (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance-associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are known, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3'

SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3'

SEQ ID NO. 2

- 5 -

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3'

SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3'

SEQ ID NO. 4

SEQ. ID. NO. 5

2161 aaaccgacga catcgaccac ttcggcaacc gccgcctgcg ~~tacggtcggc gagctgarcc~~
 2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
 2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggctcgccg
 2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaaccgcg
 2401 tgtcgggggtt gaccacaag cgccgactgt cggcgctggg gcccgggcgt ctgtcacgtg
 2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgctcga ctacggcccg atgtgccccga
 2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggtc gctgtcggtg tacgcgcggg
 2581 tcaaccgcgt cggttcacg gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3'

SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3'

SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3'

SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3'

SEQ ID NO. 9

SEQ ID NO. 10

661 gctcggcgat gagcggtaca gcggtaagcg ggatctggag aaccgcgtgg ccgcgggtgca
 721 gatggggctg atctacgtga acccgagggg gccgaacggc aaccgggacc ccatggcccg
 781 ggcggctgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc
 841 gctgatcgtc ggcggtcaca ctttcggtaa gaccatggc gccggcccg ccatctggt
 901 cggccccgaa cccgaggctg ctccgctgga gcagatggg ttgggctgga agagctcgta
 961 tggcaccgga accggttaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac
 1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac

- 6 -

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat
 1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct
 1201 gcgggtggat ccgatctatg agcggatcac ~~gcgtcgctgg ctggaacacc~~ ccgaggaatt
 1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccggtgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3'

SEQ ID NO. 11

PR-R amplification primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3'

SEQ ID NO. 12

PR-5s sequencing primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3'

SEQ ID NO. 13

PR-3s sequencing primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3'

SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc
 421 tcatatcgag aatgcttgcg gcaactgctga ~~accactgctt tgccgccacc~~ gcggcgaacg
 481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatattggtgt
 541 gatatatcac ctttgccctga cagcgacttc acggcacgat ggaatgtcgc aaccaaattgc
 601 attgtccgct ttgatgatga ggagagtcac gccactgcta accattggcg atcaattccc
 661 ~~cgcctaccag ctcaccgctc tcatcgccgg~~ tgacctgtcc aaggtcgacg ccaagcagcc
 721 cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc ggggtggtgt

mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3'

SEQ ID NO. 16

mabA-R amplification primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3'

SEQ ID NO. 17

mabA-5s sequencing primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3'

SEQ ID NO. 18

- 7 -

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3'

SEQ ID NO. 19

SEQ ID NO. 20

1 agcgcgacat acctgctgcg caattcgtag ggcgtcaata caccgcgacg cagggcctcg
 61 ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg
 121 agcgtaaccc cagtgcgaaa gttcccgcgcg gaaatcgacg ccacgttacg ctctgtggaca
 181 taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac
 241 tgaagggggc aaacccccat tcgtatcccg ttcagtcctg gttaccggag gaaaccgggg
 301 gatcgggctg gcgatcgac agcggctggc tgccgacggc cacaaggtgg ccgtcaccca

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3'

SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3'

SEQ ID NO. 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3'

SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3'

SEQ ID NO. 24

SEQ ID NO. 25

1 cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag
 61 gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgctgtac
 121 accaccactc cgaagaagcc gaactcggcg ctccggaagg ttgcccgcgt gaagttgacg
 181 agtcaggctg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg
 241 atgggtgctg tgccgcggcg ccgggtgaag gacctgctg gtgtgcgcta caagatcatc
 301 cgcggttcgc tggatacgca ggggtgtcaag aaccgcaaac aggcacgcag ccgttacggc
 361 gctaagaagg agaagggtg atgccacgca aggggcccgc gcccaagcgt ccgttggtca

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3'

SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

- 8 -

5' CGT CAC CCC ACC AAC AAG CTG 3'

SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3'

SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3'

SEQ ID NO. 29

SEQ ID NO. 30

1 cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata
61 ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg
121 gcctatcagc ttgttgggtgg ggtgacg

embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3'

SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3'

SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3'

SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3'

SEQ ID NO. 34

SEQ ID NO. 35

7741 cggcatgcmc cggtgatcc ~~cggcaagctg ggcacacccc accctgaccg~~ acgccgtggt
7801 gatattcggc ttctgtctct ggcatgtcat cggcgcgaaat tcgtcggacg acggctacat
7861 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctgggt
7921 cggcagcccc gaggatccct tcggtggtta ttacaacctg ctggcgctga tgacccatgt
7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc ~~gccgggctag tgrgctggct~~

pncA (pyrazinamide resistance)

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3'

SEQ ID NO. 36

- 9 -

pncA-F amplification primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3'

SEQ ID NO. 37

pncA-5s sequencing primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3'

SEQ ID NO. 38

pncA-3s sequencing primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3'

SEQ ID NO. 39

SEQ ID NO. 40

1 atgcgggctg tgatcatcgt cgacgtgcag aacgacttct gcgaggggtgg ctgctggcg
 61 gtaaccggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
 121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc
 181 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgccggac
 241 ttccatccca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc
 301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
 361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
 421 cagacggccg aggacgcggt acgcaatggc ttggccacca ggggtgctggt ggacctgaca
 481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
 541 gagttgggttt gcagctcctg a

gyrA (fluoroquinilone/ciprofloxacin resistance)

gyrA-F amplification primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3'

SEQ ID NO. 41

gyrA-R amplification primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3'

SEQ ID NO. 42

gyrA-5s sequencing primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3'

SEQ ID NO. 43

gyrA-3s sequencing primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3'

SEQ ID NO. 44

SEQ ID NO. 45

2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
 2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtagc
 2461 tcgccgggtg ctctatgcaa tggtcgattc cggcttccgc ccggaccgca gccacgcca

- 10 -

2521 gtcggccccg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat
 2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tgggtggacgg
 2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagg
 2701 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3'

SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3'

SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3'

SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3'

SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcggtgg taactataac catcctaagg tagcgaatt ccttgcggg
 2461 taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg
 2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccgga
 2581 ccttcactac aacttggtat tgggtgttcgg tacggtttgt gtaggatagg tgggagactt
 2641 tgaagcacag acgccagttt gtgtggagtc gttgtrgaaa taccactctg atcgtatttg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENTM sequencer), at least one of the sequencing primers is preferably labeled with a fluorescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers); and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

- 11 -

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix		1 PCR	10 PCRs	final conc. / PCR
genomic DNA	(20ng/ul)	1.0ul		20ng
(~0.5fM)				
10X PCR buffer I		2.5ul	25.0ul	1X
2.5mM dNTP mix	(1:1:1:1)	2.5ul	25.0ul	250uM
DMSO		1.3ul	13.0ul	5%
Taq DNA polymerase (1U)		0.2ul	2.0ul	1 unit
molecular grade water		16.5ul		165.0ul
MTB gene primers	(10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR		25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

- 12 -

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenace™ buffer	2.5ul

- 13 -

DMSO	3.5ul
2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
<u>1:10 diluted Thermosequenase</u>	<u>0.5 ul</u>
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

- 14 -

The CLIPPER™ sequencer is set-up as described in the *OPENGENE Automated DNA Sequencing System User Manual*. Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is its limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect *M. tuberculosis* (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat *M. tuberculosis* infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

- 15 -

and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (rpoB), isoniazid (katG), streptomycin (rpsL/s12) and azithromycin (23S). In addition the rpoB amplification indicates the presence of *M. tuberculosis* and in the absence of rpoB amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (embB), pyrazinamide (pncA) and ciprofloxacin (gyrA). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada, Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the rpsL/s12 gene in four isolates. Parallel antibiotic resistance-associated mutations in the rpoB (rifampin), katG (isoniazid), PR (isoniazid),

- 16 -

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

- ^a DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 38: 2380-2386.
- ^b WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *Antimicrob Agents Chemother* 41: 1601-1603.
- ^c S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. *Antimicrob Agents Chemother* 41: 600-606.
- ^d A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at the reference laboratory level. *Antimicrob Agents Chemother* 35: 719-723.
- ^e C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- ^f C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- ^g MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 41: 2629-2633.
- ^h A Scorpio et al. (1997). Characterisation of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 41: 540-543.

- 17 -

C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *J Infect Disease* 174: 1127-1130.

KA Nash et al. (1995). Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. *Antimicrob Agents Chemother* 39: 2625-2630.

- 17/1 -

Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553ltg, Ser553Leu	cac526gac, His526Asp	tcg553ltg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	aag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	aag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	glc292tlc, val292phe	wt	wt	wt
pncA (pyrazinamide)	lcc65lcl, Ser65Ser	wt	alt133aat, Ile133Asn	wt	lcc65lcl, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:
 - (a) obtaining a sputum sample suspected of containing *M. tuberculosis*,
 - (b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the *rpoB*, *katG*, *rpsL/s12* and *23S* genes for the presence of antibiotic-resistance inducing mutations; and
 - (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.
2. The method of claim 1, wherein the second sequencing procedure evaluates *PR*, *embB*, *pncA* and *gyrA* genes for the presence of antibiotic-resistance mutations.
3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate *16S/rrs* and *mabA* genes for the presence of antibiotic-resistance mutations.
4. The method of any of claims 1 to 3, wherein the first sequencing procedure for *rpoB* is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.
5. The method of any of claims 1 to 4, wherein the first sequencing procedure for *katG* is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.
6. The method of any of claims 1 to 5, wherein the first sequencing procedure for *rpsL/s12* is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

- 19 -

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.

8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.

9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.

10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.

11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.

12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.

13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.

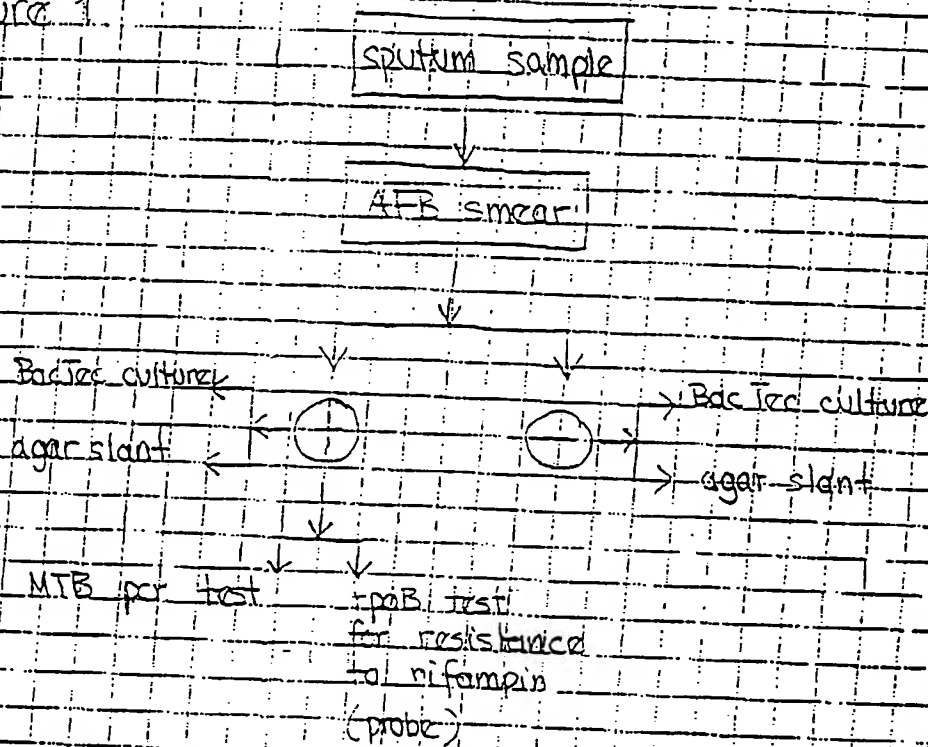
14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

- 20 -

the genome of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs are selected from among:

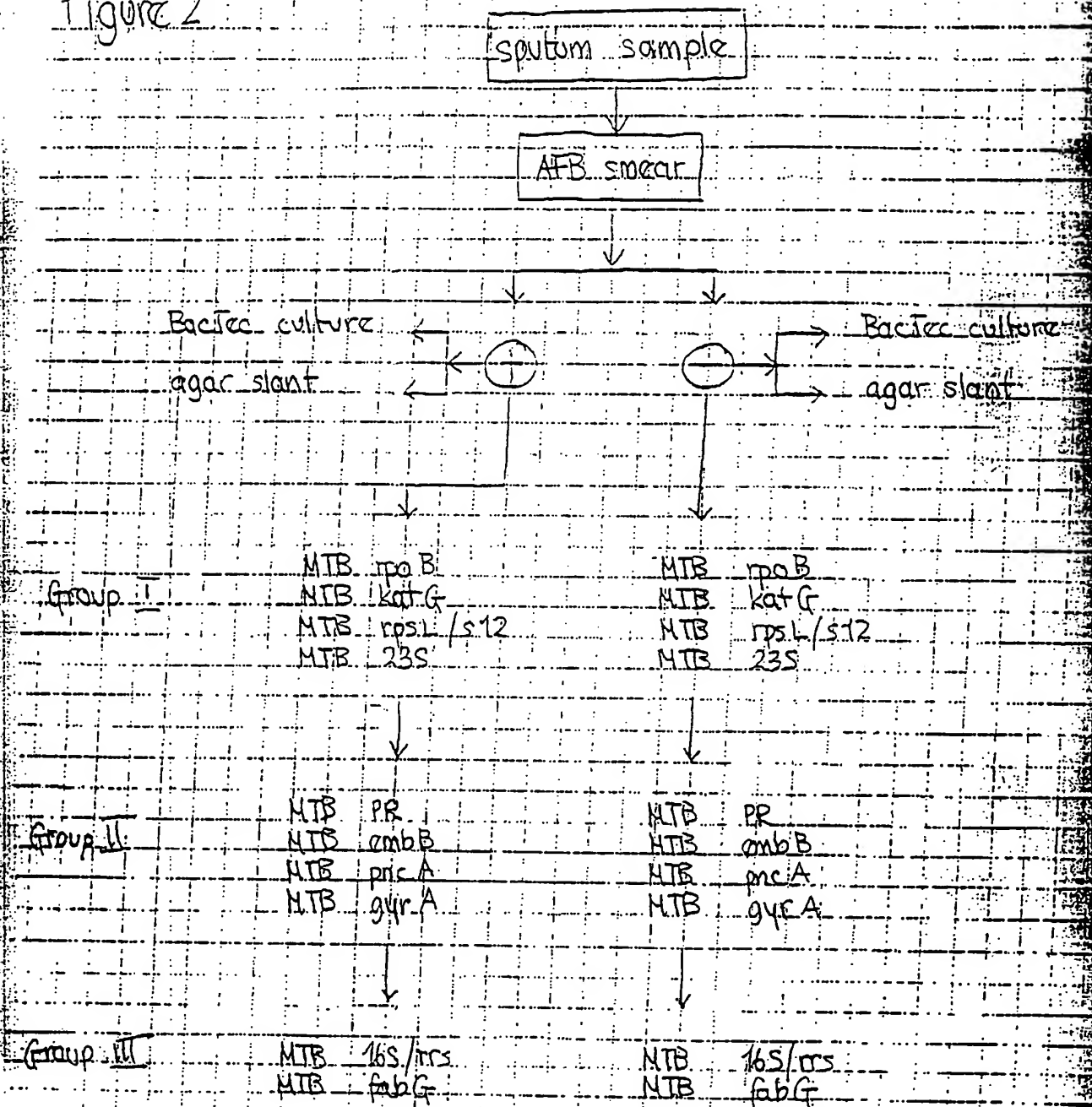
- (a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;
- (b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;
- (c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;
- (d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;
- (e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;
- (f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;
- (g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;
- (h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;
- (i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and
- (j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

Figure 1



2/2

Figure 2



SEQUENCE LISTING¹

<110> Visible Genetics Inc.
Shipman, Robert

<120> Method and Kit for the Characterization of
Antibiotic-Resistance Mutations in Mycobacterium
tuberculosis

<130> VGEN.P-055-WO

<140>

<141>

<150> 60/111,794

<151> 1998-12-11

<160> 50

<170> PatentIn Ver. 2.1

<210> 1

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-F amplification primer

<400> 1

tacggtcggc gagctgatcc

20

<210> 2

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-R amplification primer

<400> 2

tacggcggtt cgatgaaccc

20

<210> 3
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB-5s sequencing primer

<400> 3
tacggtcggc gagctgatcc 20

<210> 4
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB-3s sequencing primer

<400> 4
tacggcgttt cgtgaaccc 20

<210> 5
<211> 480
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpoB (rifampin resistance)

<400> 5
aaaccgacga catcgaccac ttggcaacc gccgcctgcg tacggtcggc gagctgatcc 60
aaaaccagat cggggtcggc atgtcgcgga tggagcgggt ggtccgggag cggtatgacca 120
cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg 180
cgtcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaaccgc 240
tgtcgggggtt gaccacaag cgccgactgt cggcgctggg gcccggcggt ctgtcacgtg 300
agcgtgccgg gctggaggtc cgcgacgtgc accgctcgca ctacggccgg atgtgcccga 360
tcgaaacccc tgagggggccc aacatcggtc tgatcggctc gctgtcgggtg tacgcgcggg 420
tcaaccgtt cgggttcacg gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg 480

<210> 6
<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> katG-F amplification primer

<400> 6

atggggctga tctacgtgaa

20

<210> 7

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> katG-R amplification primer

<400> 7

ggtgttccag ccagcgacgc

20

<210> 8

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> katG-5s sequencing primer

<400> 8

atggggctga tctacgtgaa

20

<210> 9

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> katG-3s sequencing primer

<400> 9

ggtgttccag ccagcgacgc

20

<210> 10
<211> 660
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG (isoniazid resistance)

<400> 10
gctcggcgat gagegttaca gcggttaagcg ggatctggag aacccgctgg ccgcggtgca 60
gatggggctg atctacgtga acccggaggg gccgaacggc aacccggacc ccatggccgc 120
ggcggtcgac attcgcgaga cgttcggcg catggccatg aacgacgtcg aaacagcggc 180
gctgatcgtc ggcggtcaca ctttcggtaa gacctatggc gccggcccgg ccgatctggt 240
cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta 300
tggcaccgga accggttaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac 360
cccgcgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac 420
gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat 480
cccgagcccg ttggcggggc cagggcgctc cccgacgatg ctggccactg acctctcgct 540
gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt 600
ggccgacgag ttgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgtgc 660

<210> 11
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-F amplification primer

<400> 11
accactgctt tgccgccacc 20

<210> 12
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-R amplification primer

<400> 12
ccgatgagag cggtgagctg 20

<210> 13
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-5s sequencing primer

<400> 13
accactgctt tgccgccacc 20

<210> 14
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-3s sequencing primer

<400> 14
ccgatgagag cggtagctg 20

<210> 15
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> oxyR-ahpC intergenic region (PR)

<400> 15
atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc 60
tcatacgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg 120
cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt 180
gatatacac ctttgctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc 240
attgtccgct ttgatgatga ggagagtcac gccactgcta accattggcg atcaattccc 300
cgcctaccag ctcaccgctc tcacggcgcg tgacctgtcc aaggtcgacg ccaagcagcc 360
cggcgactac ttaccacta tcaccagtga cgaacacca ggcaagtggc ggggtggtgtt 420

<210> 16
<211> 20
<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-F amplification primer

<400> 16

cctcgctgcc cagaaaggga

20

<210> 17

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-R amplification primer

<400> 17

atcccccggt ttctccggt

20

<210> 18

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-5s sequencing primer

<400> 18

cctcgctgcc cagaaaggga

20

<210> 19

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-3s sequencing primer

<400> 19

atcccccggt ttctccggt

20

<210> 20
<211> 360
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG (isoniazid resistance)

<400> 20
agcgcgacat acctgctgcg caattcgtag ggcgtcaata caccgcgagc cagggcctcg 60
ctgcccagaa agggatccgt catggcgaa gtgtgctgag tcacaccgac aaacgtcacg 120
agcgtaaccc cagtgcgaaa gttcccgcg gaaatcgag ccacgttacg ctctggaca 180
taccgatttc ggcccggccg cggcgagacg ataggttgtc ggggtgactg ccacagccac 240
tgaaggggcc aaaccccat tcgtatccg ttcagtcctg gttaccggag gaaaccgggg 300
gatcgggctg gcgatgcac agcgggtggc tgccgacggc cacaaggtgg ccgtcaccca 360

<210> 21
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-F amplification primer

<400> 21
cggtagatgc caaccatcca 20

<210> 22
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-R amplification primer

<400> 22
gcatcagccc ttctcttct 20

<210> 23
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>

<223> s12-5s sequencing primer

<400> 23

cggtagatgc caaccatcca

20

<210> 24

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> s12-3s sequencing primer

<400> 24

gcatcagccc ttctccttct

20

<210> 25

<211> 420

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpsL/s12 (streptomycin resistance)

<400> 25

cggtagatgc caaccatcca gcagctgggc cgcaaggggc gtcgggacaa gatcagtaag 60
gtcaagaccg cggtctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgcgtgtac 120
accaccactc cgaagaagcc gaactcggcg ctccggaagg ttgccgcgt gaagttgacg 180
agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg 240
atgggtgctgg tgcgcggcgg ccgggtgaag gacctgcctg gtgtgcgcta caagatcatc 300
cgcggttcgc tggatacgca ggggtgtcaag aaccgcaaac aggcacgcag ccgttacggc 360
gctaagaagg agaagggctg atgccacgca aggggcccgc gccaagcgt ccgttggtca 420

<210> 26

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S-F amplification primer

<400> 26

ggtgatctgc cctgcacttc g

21

<210> 27

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S-R amplification primer

<400> 27

cgtcacccca ccaacaagct g

21

<210> 28

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S-5s sequencing primer

<400> 28

ggtgatctgc cctgcacttc g

21

<210> 29

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S-3s sequencing primer

<400> 29

cgtcacccca ccaacaagct g

21

<210> 30

<211> 147

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S/rrs (streptomycin resistance)

<400> 30

cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata 60
ggaccacggg atgcatgtct tgtggtggaa agcgcttag cgggtgtggga tgagcccgcg 120
gcctatcagc ttgttggtgg ggtgacg 147

<210> 31

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-F amplification primer

<400> 31

cggcaagctg ggcaccttc a 21

<210> 32

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-R amplification primer

<400> 32

agccagcaca ctagcccggc g 21

<210> 33

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-5s sequencing primer

<400> 33

cggcaagctg ggcaccttc a 21

<210> 34
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB-3s sequencing primer

<400> 34
agccagcaca ctagcccggc g

21

<210> 35
<211> 300
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> embB (ethambutol resistance)

<400> 35
cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggt 60
gatattcggc ttctgtctt ggcatgtcat cggcgcgaat tcgtcggacg acggctacat 120
cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgtggtt 180
cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacctatgt 240
cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct 300

<210> 36
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 36
atgcgggcgt tgatcatcgt

20

<210> 37
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>

<223> pncA-F amplification primer

<400> 37

tcaggagctg caaaccaact

20

<210> 38

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> pncA-5s sequencing primer

<400> 38

atgcgggcgt tgatcatcgt

20

<210> 39

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> pncA-3s sequencing primer

<400> 39

tcaggagctg caaaccaact

20

<210> 40

<211> 561

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> pncA (pyrazinamide resistance)

<400> 40

atgcgggcgt tgatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctgctggcg 60
gtaaccgggtg gcgccgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac 120
taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca ttctccggc 180
acaccggact attctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
ttccatcca gtctggacac gtcggcaatc gaggcggtgt tctacaaggg tgcctacacc 300

ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg 360
 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc 420
 cagacggccg aggacgcggt acgcaatggc ttggccacca ggggtgctggt ggacctgaca 480
 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc 540
 gagttggtt gcagctcctg a 561

<210> 41
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-F amplification primer

<400> 41
 cagctacatc gactatgcga 20

<210> 42
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-R amplification primer

<400> 42
 gggcttcggt gtacctcatc 20

<210> 43
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-5s sequencing primer

<400> 43
 cagctacatc gactatgcga 20

<210> 44
 <211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> gyrA-3s sequencing primer

<400> 44

gggcttcggt gtacctcatc

20

<210> 45

<211> 420

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> gyrA (fluoroquinilone/ciprofloxacin resistance)

<400> 45

cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc 60
gatgagcgtg atcgtcggcc gcgcgctgcc ggagggtcgc gacgggctca agcccgtgca 120
tcgccgggtg ctctatgcaa tgttcgattc cggttccgc ccggaccgca gccacgcaa 180
gtcgcccggtg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat 240
ctacgacagc ctggtgcgca tggcccagcc ctggtcgtg cgctaccgc tgggtggacgg 300
ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc 360
ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt 420

<210> 46

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-F amplification primer

<400> 46

cgaaattcct tgcgggtaa

20

<210> 47

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-R amplification primer

<400> 47

gtatttcaac aacgactcca

20

<210> 48

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-5s sequencing primer

<400> 48

cgaaattcct tgcgggtaa

20

<210> 49

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-3s sequencing primer

<400> 49

gtatttcaac aacgactcca

20

<210> 50

<211> 300

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S (macrolide/azithromycin resistance)

<400> 50

gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt cctgtcggg 60
taagttccga cctgcacgaa tggcgtaacg acttccaac tgtctcaacc atagactcgg 120
cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccgga 180
ccttcactac aacttggtat tgggttcgg tacggttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgtgaaa taccactctg atcgattgg 300

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract D71 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document --- -/-	1-3

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

13 April 2000

Date of mailing of the international search report

28/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS: A MOLECULAR STUDY" LANCET THE, GB, LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ; LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ; HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ; BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3
	--- -/--	

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM"</p> <p>ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13</p> <p>-----</p>	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 99/01177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723650 A	03-07-1997	US 5834189 A	10-11-1998
		AU 1426297 A	17-07-1997
		CA 2239896 A	03-07-1997
		EP 0870059 A	14-10-1998
		WO 9741257 A	06-11-1997
		US 5888736 A	30-03-1999
		US 5981186 A	09-11-1999
WO 9533074 A	07-12-1995	US 6007983 A	28-12-1999
WO 9533074 A	07-12-1995	US 5643723 A	01-07-1997
		AU 2605795 A	21-12-1995
WO 9533851 A	14-12-1995		
		AU 703947 B	01-04-1999
		AU 2789695 A	04-01-1996
		BR 9507960 A	02-09-1997
		CZ 9603612 A	16-07-1997
		EP 0771360 A	07-05-1997
		JP 10500857 T	27-01-1998

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 June 2000 (22.06.2000)

PCT

(10) International Publication Number
WO 00/36142 A1

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number: PCT/CA99/01177

(22) International Filing Date:
10 December 1999 (10.12.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/111,794 11 December 1998 (11.12.1998) US

(71) Applicant (for all designated States except US): **VISIBLE GENETICS INC.** [CA/CA]; 700 Bay Street, P.O. Box 333, Toronto, Ontario M5G 1Z6 (CA).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **SHIPMAN, Robert** [CA/CA]; #2-6240 Montevideo Road, Mississauga, Ontario L5N 2N7 (CA).

(74) Agent: **DONAHUE ERNST & YOUNG**; Ernst & Young Tower, Suite 1800, 222 Bay Street, P.O. Box 197, T.D. Centre, Toronto, Ontario M5K 1H6 (CA).

(81) Designated States (*national*): AU, CA, JP, US.

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:

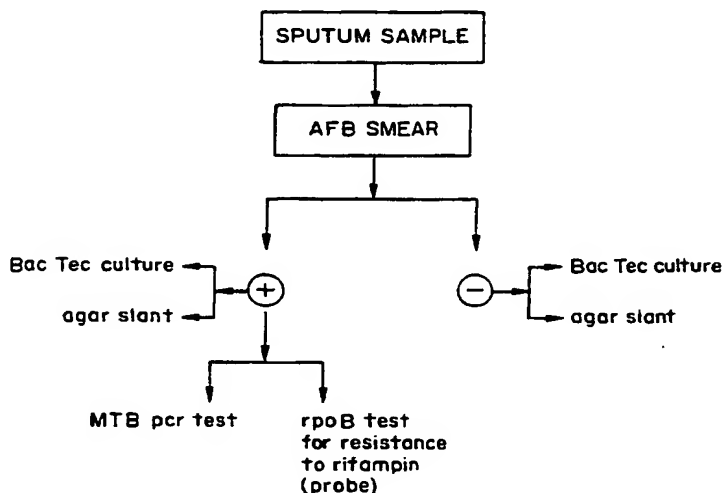
— With international search report.

(48) Date of publication of this corrected version:
12 July 2001

(15) Information about Correction:
see PCT Gazette No. 28/2001 of 12 July 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS*



(57) Abstract: Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the *rpoB* (rifampin), *katG* (isoniazid), *oxyR-ahpC PR* (isoniazid), *mabA* (isoniazid), *rpsL/s12* (streptomycin), *16S/rrs* (streptomycin), *embB* (ethambutol), *pncA* (pyrazinamide), *gyrA* (ciprofloxacin) and *23S* (azithromycin) genes of *Mycobacterium tuberculosis*. These primers can be used in a method for detection and characterization of *Mycobacterium tuberculosis* present in a sample. The method includes the steps of obtaining a sputum sample suspected of containing *M. tuberculosis*, performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the *rpoB*, *katG*, *rpsL/s12* and *23S* genes for the presence of antibiotic-resistance inducing mutations; and (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.

WO 00/36142 A1

METHOD AND KIT FOR THE CHARACTERIZATION OF ANTIBIOTIC-RESISTANCE MUTATIONS IN MYCOBACTERIUM TUBERCULOSIS

DESCRIPTION

Field of the Invention

This application relates to a method and kit for the characterization of antibiotic resistance mutations in *Mycobacterium tuberculosis*, and particularly to the evaluation of such mutations in clinical samples.

Background of the Invention

M. tuberculosis can be resistant to all antibiotics that are currently used to treat tuberculosis patients. Antibiotic resistance is due to acquired point mutations in target genes in the genome of *M. tuberculosis*. These point mutations render the organism insensitive to the action of the antibiotic by preventing its uptake or activation, or by altering the antibiotic target. The observed antibiotic resistance in *M. tuberculosis* is not due to an episome-encoded resistance gene transferred from one strain to another and, like other bacteria, is single-step (one point mutation), high-level resistance.

Rapid and accurate detection of antibiotic resistance in *Mycobacterium tuberculosis* in sputum samples would greatly improve both patient treatment and outcome. Presently, analysis of *M. tuberculosis* is carried out on DNA recovered from sputum samples handled according to Standard Infectious Disease/Public Health Laboratory practices. The sputum sample is decontaminated and a cell sediment isolated. This cell sediment is the sample source for all routine procedures used in the detection and isolation of *M. tuberculosis*. Portions of this sample are used in BacTec cultures for selective growth of *M. tuberculosis*, agar plate/agar slant cultures for *M. tuberculosis*, acid-fast bacilli (AFB) smears for mycobacteria detection and molecular biological methods for the detection of *M. tuberculosis* and atypical mycobacteria. (See Fig. 1)

Mycobacterial DNA is prepared directly from the decontaminated sputum cell sediments according to standard procedures and this mycobacterial DNA is used in the various molecular biological detection procedures. The methods presently in use for the detection of *M. tuberculosis* are either PCR-based or probe-based. These tests are used

primarily on AFB smear-positive samples. Since the presence of *M. tuberculosis* has already been established by the AFB smear, these tests are used primarily in a confirmatory capacity as opposed to a diagnostic capacity. Furthermore, these tests provide no information on the potential antibiotic resistance of these *M. tuberculosis* samples.

Below is a list of antibiotics used to treat *M. tuberculosis* infections. The gene target of the specific antibiotic and regions associated with antibiotic resistance are listed, if known. The references on which the codon assignments are based are listed at the end of the application.

1.	Rifampin	rpoB gene	codon 507-533a
2.	Isoniazid	katG gene	codon 275/315/328b
3.	Isoniazid	mabA gene	unknown c
4.	Isoniazid	oxyR-ahpC intergenic region (PR)	nucleotides -48 to +33
5.	Azithromycin	23S rRNA sequence	nucleotide 2568A e
6.	Pyrazinamide	pncA gene	codon 47/85 f
7.	Ethambutol	embB gene	codon 306 g
8.	Streptomycin	rpsL/s12 gene	codon 43/88 h
9.	Streptomycin	16S/rrs sequence	nucleotides 491, 512, 516, 513, 903, 904
10.	Ciprofloxacin	gyrA gene	codon 88-95 j

Probe-based tests do exist for the determination of rifampin resistance in *M. tuberculosis* (line probe assay-InnoTek), but these probes rely on prior knowledge of antibiotic resistance-associated mutations in the rpoB gene. Mutations outside the region covered by the probe or new mutations not included in the probe cocktail could still confer resistance, but would not be detected using this product in its present form.

Thus, there remains a need for a method for detecting antibiotic-resistance mutations in clinical *M. tuberculosis* sputum samples which is capable of detecting mutations in all of the gene targets which confer antibiotic resistance. It is an object of the present invention to provide such a method. It is a further object of this invention to provide amplification and cycle sequencing primer sets, and kits containing such primer sets, for use in the characterization of antibiotic resistance mutations in *M. tuberculosis*.

Summary of Invention

Amplification and cycle sequencing primer sets have been developed for the detection and analysis of antibiotic resistance-associated mutations in defined regions of the *rpoB* (rifampin), *katG* (isoniazid), *oxyR-ahpC* PR (isoniazid), *mabA* (isoniazid), *rpsL/s12* (streptomycin), *16S/rrs* (streptomycin), *embB* (ethambutol), *pncA* (pyrazinamide), *gyrA* (ciprofloxacin) and *23S* (azithromycin) genes. Using these primer sets and the OPENGENE™ automated DNA sequencing system, a protocol has been developed which permits both the rapid detection of *M. tuberculosis* and the identification of antibiotic resistance-associated mutations in a series of gene targets. The present invention uses a series of tests designed to detect antibiotic resistance-associated mutation in all gene targets for all antibiotics presently in use for the treatment of tuberculosis. The tests are employed in a hierarchical manner on both AFB smear-positive or smear-negative samples to determine both the presence and antibiotic-resistance of *M. tuberculosis* in a given sample. This method permits the simultaneous determination of *M. tuberculosis* presence in a sample and the antibiotic-resistance profile to an entire panel of antibiotics. Standard methods require from 2-6 weeks to culture *M. tuberculosis* and additional time to establish antibiotic resistance. Although DNA sequence-based (genotypic) tests are not intended to replace the traditional culture-based (phenotypic) methods, these tests do represent a rapid, sensitive and accurate protocol which provides clinicians with valuable information regarding antibiotic treatment options within days as opposed to weeks.

Brief Description to the Figures

Fig. 1 shows known testing protocols for *M. tuberculosis*; and

Fig. 2 shows a hierarchical assay scheme for evaluating *M. tuberculosis* type in accordance with the invention.

Detailed Description of the Invention

In accordance with the invention, regions of the genome of *M. tuberculosis* associated with antibiotic resistance are amplified and sequenced using specifically designed amplification and sequencing primers. Various techniques for amplification are known, including the basic PCR amplification techniques described in US Patent No. 4,683,202, which is incorporated herein by reference. Similarly, various techniques for sequencing are known, some of which require prior amplification and some of which do not. Included among known sequencing techniques are those disclosed in US Patents Nos. 5,834,189 and 5,789,168, which are incorporated herein by reference. The primers of the invention can be used in any of these sequencing formats, although the invention is exemplified below using separate amplification and cycle-sequencing steps.

In theory, the selection of primers to amplify and sequence a known region of interest should be straightforward. In fact, however, because of the possibility of primer binding to other sites, complications arising from secondary structure, and other factors which are not fully understood, some primers perform better than others for amplification and sequencing of the same region of interest. The present invention provides primers which have been optimized for the amplification and sequencing of regions associated with each of the ten known types of antibiotic resistance. These primer sets are shown below, along with the sequence of the genes that they are used to analyze. In the gene sequences, the locations of the primers are underlined.

Primers

rpoB (rifampin resistance)

rpoB-F amplification primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3'

SEQ. ID NO. 1

rpoB-R amplification primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3'

SEQ ID NO. 2

5

rpoB-5s sequencing primer, 20-mer, bp2201-2220

5' TAC GGT CGG CGA GCT GAT CC 3'

SEQ ID NO. 3

rpoB-3s sequencing primer, 20-mer, bp2611-2592

5' TAC GGC GTT TCG ATG AAC CC 3'

SEQ ID NO. 4

SEQ. ID. NO. 5

2161 aaaccgacga catcgaccac ttggcaacc gccgcctgcg tacggtcggc gagctgatcc
 2221 aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca
 2281 cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg
 2341 cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaaccgc
 2401 tgtcgggggt gaccacaag cggcgactgt cggcgctggg gccggcggt ctgtcacgtg
 2461 agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgccga
 2521 tcgaaacccc tgaggggccc aacatcggtc tgatcggctc gctgtcgggt tacgcgcggg
 2581 tcaaccggt cgggttcac gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg

katG (isoniazid resistance)

katG-F amplification primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3'

SEQ ID NO. 6

katG-R amplification primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3'

SEQ ID NO. 7

katG-5s sequencing primer, 20-mer, bp722-741

5' ATG GGG CTG ATC TAC GTG AA 3'

SEQ ID NO. 8

katG-3s sequencing primer, 20-mer, bp1250-1231

5' GGT GTT CCA GCC AGC GAC GC 3'

SEQ ID NO. 9

SEQ ID NO. 10

661 gtcggcgat gagcgttaca gcggttaagcg ggatctggag aaccgcctgg ccgcggtgca
 721 gatggggctg atctacgtga acccggaggcg gccgaacggc aaccgggacc ccatggccgc
 781 ggcggtcgac attcgcgaga cgttcggcg catggccatg aacgacgtc aaacagcggc
 841 gctgacgtc ggcggtcaca ctccggtaa gaccatggc gccggccggg ccgatctggt
 901 cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgtg
 961 tggcaccgga accggttaagg acgcgatcac cagcggcatc gaggtcgtat ggacgaacac
 1021 cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac

6

1081 gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgccggtg ccggcaccat
 1141 cccggacccg ttcggcgggc cagggcgctc cccgacgatg ctggccactg acctctcgct
 1201 gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt
 1261 ggccgacgag ttcgccaagg cctggtacaa gctgatccac cgagacatgg gtcccgttgc

oxyR-aphC intergenic region (PR)

PR-F amplification primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3'

SEQ ID NO. 11

PR-R amplification primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3'

SEQ ID NO. 12

PR-5s sequencing primer, 20-mer, bp451-470

5' ACC ACT GCT TTG CCG CCA CC 3'

SEQ ID NO. 13

PR-3s sequencing primer, 20-mer, bp687-668

5' CCG ATG AGA GCG GTG AGC TG 3'

SEQ ID NO. 14

SEQ ID NO. 15

361 atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc
 421 tcatatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg
 481 cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt
 541 gatatatcac cttgcctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc
 601 attgtccgct ttgatgatga ggagagtcac gccactgcta accattggcg atcaattccc
 661 cgcctaccag ctcaccgctc tcacggcg tgacctgtcc aaggtcgacg ccaagcagcc
 721 cggcgactac ttcaccata tcaccagtga cgaacacca ggcaagtggc gggtggtgtt
mabA (isoniazid resistance)

mabA-F amplification primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3'

SEQ ID NO. 16

mabA-R amplification primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3'

SEQ ID NO. 17

mabA-5s sequencing primer, 20-mer, bp56-75

5' CCT CGC TGC CCA GAA AGG GA 3'

SEQ ID NO. 18

SUBSTITUTE SHEET (RULE 26)

7

mabA-3s sequencing primer, 20-mer, bp303-284

5' ATC CCC CGG TTT CCT CCG GT 3'

SEQ ID NO. 20

SEQ ID NO. 19

1 agcgcgacat accctgctgcg caattcgtag ggcgtcaata caccgcgacg cagggcctcg
61 ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg
121 agcgttaaccc cagtgcgaaa gtccccgccg gaaatcgag ccacgttacg ctctgggaca
181 taccgatttc ggcccgcccg cggcgagacg ataggttgc ggggtgactg ccacagccac
241 tgaaggggcc aaaccccat tcgtatcccg ttacgtcctg gtaccggag gaaaccgggg
301 gatcgggctg gcgatgcac agcggctggc tgccgacggc cacaagggtg ccgtcaccca

rpsL/s12 (streptomycin resistance)

s12-F amplification primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3'

SEQ ID NO. 21

s12-R amplification primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3'

SEQ ID NO. 22

s12-5s sequencing primer, 20-mer, bp1-20

5' CGG TAG ATG CCA ACC ATC CA 3'

SEQ ID NO. 23

s12-3s sequencing primer, 20-mer, bp384-365

5' GCA TCA GCC CTT CTC CTT CT 3'

SEQ ID NO. 24

SEQ ID NO. 25

1 cggtagatgc caaccatcca gcagctggtc cgcaagggtc gtcgggacaa gatcagtaag
61 gtcaagaccg cggctctgaa gggcagcccc cagcgtcgtg gtgtatgcac ccgcgtgtac
121 accaccactc cgaagaagcc gaactcggcg cticgggaagg ttgcccgct gaagttgacg
181 agtcaggctg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg
241 atggtgctgg tgcgcggcgg cgggtgaag gacctgcctg gtgtgcgcta caagatcatc
301 cgcggttcgc tggatacgca ggggtgcaag aaccgcaaac aggcacgcag ccgttacggc
361 gctaagaagg agaagggtg atgccacgca aggggcccgc gcccaagcgt ccgttggtca

16S/rrs (streptomycin resistance)

16S-F amplification primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3'

SEQ ID NO. 26

16S-R amplification primer, 21-mer, bp147-127

8

5' CGT CAC CCC ACC AAC AAG CTG 3'

SEQ ID NO. 27

16S-5s sequencing primer, 21-mer, bp5-25

5' GGT GAT CTG CCC TGC ACT TCG 3'

SEQ ID NO. 28

16S-3s sequencing primer, 21-mer, bp147-127

5' CGT CAC CCC ACC AAC AAG CTG 3'

SEQ ID NO. 29

SEQ ID NO. 30

1 cgtgggtgat ctgccctgca ctctgggata agcctgggaa actgggtcta ataccggata

61 ggaccacggg atgcatgtct tgtgtggaa agcgcttag cgggtggga tgagcccgcg

121 gcctatcagc ttgttggtgg ggtgacg

embB (ethambutol resistance)

embB-F amplification primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3'

SEQ ID NO. 31

embB-R amplification primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3

SEQ ID NO. 32

embB-5s sequencing primer, 21-mer, bp7761-7781

5' CGG CAA GCT GGC GCA CCT TCA 3'

SEQ ID NO. 33

embB-3s sequencing primer, 21-mer, bp8040-8020

5' AGC CAG CAC ACT AGC CCG GCG 3

SEQ ID NO. 34

SEQ ID NO. 35

7741 cggcatgcgc cggctgattc cggcaagctg ggcgcacctc accctgaccg acgccgtggt

7801 gatattcggc ttctgtctt ggcattgcat cggcgcgaat tcgtcggacg acggctacat

7861 cctgggcatg gcccagatcg ccgaccacgc cggctacatg tccaactatt tccgtggtt

7921 cggcagcccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt

7981 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc ggcgggctag tgtcctggct**pncA (pyrazinamide resistance)**

pncA-F amplification primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3'

SEQ ID NO. 36

pncA-F amplification primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3'

SEQ ID NO. 37

pncA-5s sequencing primer, 20-mer, bp1-20

5' ATG CGG GCG TTG ATC ATC GT 3'

SEQ ID NO. 38

pncA-3s sequencing primer, 20-mer, bp561-542

5' TCA GGA GCT GCA AAC CAA CT 3'

SEQ ID NO. 39

SEQ ID. NO. 40

1 atgcggggcgt tcatcatcgt cgacgtgcag aacgacttct gcgagggtgg ctgcctggcg
61 gtaaccgggtg gcggcgcgct ggcccgcgcc atcagcgact acctggccga agcggcggac
121 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca ctctccggc
181 acaccggact attctcgtc gtggccaccg cattgcgtca gcggctactcc cggcgcggac
241 ttcatccca gtctggacac gtcggcaatc gaggcgggtgt tctacaaggg tgcctacacc
301 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgccactgct gaattggctg
361 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc
421 cagacggccc aggacgcggt acgcaatggc ttggccacca ggggtgctggt ggacctgaca
481 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc
541 gagttggtt gcagctcctg a

gyrA (fluoroquinilone/ciprofloxacin resistance)

gyrA-F amplification primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3'

SEQ ID NO. 41

gyrA-R amplification primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3'

SEQ ID NO. 42

gyrA-5s sequencing primer, 20-mer, bp2383-2402

5' CAG CTA CAT CGA CTA TGC GA 3'

SEQ ID NO. 43

gyrA-3s sequencing primer, 20-mer, bp2702-2683

5' GGG CTT CGG TGT ACC TCA TC 3'

SEQ ID NO. 44

SEQ ID NO. 45

2341 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc
2401 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccggtga
2461 tcgccgggtg cctatgcaa ttttcgattc cggcttccgc ccggaccgca gccacgcca

2521 gtcggccccg tcggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat

2581 ctacgacagc ctggtgcgca tggcccagcc ctggtcgctg cgctacccgc tgggtggacgg

2641 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc

2701 ccggctgacc ccgttggcga tggagatgct gagggaaaac gacgaggaga cagtcgatt

23S (macrolide/azithromycin resistance)

23S-F amplification primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3'

SEQ ID NO. 46

23S-R amplification primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3'

SEQ ID NO. 47

23S-5s sequencing primer, 20-mer, bp2444-2463

5' CGA AAT TCC TTG TCG GGT AA 3'

SEQ ID NO. 48

23S-3s sequencing primer, 20-mer, bp2683-2664

5' GTA TTT CAA CAA CGA CTC CA 3'

SEQ ID NO. 49

SEQ ID NO. 50

2401 gccccagtaa acggcgggtg taactataac catcctaagg tagcgaaatt ccttgcggg

2461 taagtccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg

2521 cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccgga

2581 ccttcactac aacttggtat tgggttcgg tacggttctg gtaggatagg tgggagactt

2641 tgaagcacag acgccagttt gtgtgagtc gttttgaaa taccactctg atcgattgg

To facilitate detection of the sequencing products using real-time fluorescence-based electrophoresis apparatus (for example, a Visible Genetics OPENGENE™ sequencer), at least one of the sequencing primers is preferably labeled with a fluorescent label. The label is selected for compatibility with the sequencing apparatus employed, and may be, for example, fluorescein or a cyanine dye such as CY5.0 OR CY5.5.

The primers of the invention are suitably packaged in a kit. This kit will contain individually packaged amplification and sequencing primers sets for each resistance gene to be evaluated by the kit. Thus, the kit of the invention includes at least 4 primers (two amplification and two sequencing primers), and preferably includes the primer sets for a plurality of resistance genes, most preferably the primer sets for all ten resistance genes.

The suitable protocol for the utilization of these primer sets in the evaluation of *M. tuberculosis* in clinical samples utilizes PCR amplification, followed by cycle sequencing. DNA for use in the test is obtained from a sample of sputum (100ul-10ml). The sputum sample is processed according to Standard Infectious Disease/Public Health Laboratory practices (Mycobacteriology Bench Manual, Laboratory Services Branch, December 1997, Ontario Ministry of Health). The sputum sample is homogenized, decontaminated and concentrated. Mycobacterial DNA is prepared directly from a portion of the concentrated cell sediment (100-200ul) using standard DNA extraction methods or commercially available kits.

Amplification of the DNA is performed using the amplification primer sets described above. PCR reagents can be prepared for individual reactions, or may be prepared as a master mix which can be used for multiple tests e.g., 10 PCR reactions. Exemplary combinations of reagents are summarized in the following table.

PCR mix	1 PCR	10 PCRs	final conc. / PCR
genomic DNA (20ng/ul)	1.0ul		20ng (~0.5fM)
10X PCR buffer I	2.5ul	25.0ul	1X
2.5mM dNTP mix (1:1:1:1)	2.5ul	25.0ul	250uM
DMSO	1.3ul	13.0ul	5%
Taq DNA polymerase (1U)	0.2ul	2.0ul	1 unit
molecular grade water	16.5ul	165.0ul	
MTB gene primers (10uM)	1.0ul	10.0ul	10pmol per primer
total volume per PCR	25.0ul		

If the master mix as shown in the column labeled 10 PCRs is utilized, the mastermix contains all the necessary PCR reagents other than the genomic DNA. In this example, 24.0ul of the mastermix is added to a PCR tube, that already contains 1.0ul of genomic DNA, prior to the addition of the mineral oil overlay and placement in the thermocycler.

The genomic DNA preparation utilized must be of sufficient quality and integrity for robust and reproducible PCR. Suitable DNA preparation can be obtained using

the Gentra Puregene™ DNA isolation kit. The kit components are appropriate for the isolation of genomic DNA from blood, fresh or frozen tissue, archival material and paraffin-embedded tissue.

Each primer pair is used to amplify a single gene region under the following conditions:

1.	Denaturation	94°C	5 minutes	1 cycle
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35 cycles
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1 cycle
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

After amplification, 2.0ul from the 25.0ul PCR is analysed for purity on a 0.8% agarose gel. Samples displaying single PCR product bands can be used directly for sequence analysis. The yield and purity of the PCR product determines the amount to be used in the subsequent cycle sequencing reaction. Comparable verification of sequencing purity is performed on each of the other amplification products.

Sequence analysis is carried out on the amplified product. The basic procedures and conditions are the same for each region. Accordingly, the invention will be exemplified using the rpoB gene.

For initial sequence analysis of rpoB, the rpoB-5s primer should be used. For confirmatory sequence analysis the rpoB-3s primer should be used. For each template to be sequenced, aliquot 3.0ul of each of the nucleotide termination mixes into four separate tubes marked <A>, <C>, <G> and <T> and store on ice until the sequencing mastermix is prepared.

Cycle sequencing mastermix

rpoB template	2.0ul
10X VGI Sequenace (tm) buffer	2.5ul

DMSO	13 3.5ul
2.5uM dye-sequencing primer	2.0ul
PCR grade water	9.0ul
<u>1:10 diluted Thermosequenase</u>	<u>0.5 ul</u>
total volume	22.0ul

Mix the DMSO and other components in the mastermix well by repeated pipetting (5 times) with a micropipette. Store the mastermix on ice until ready to add to the nucleotide termination mixes.

Add 5.0ul of the mastermix to each of the four marked tubes containing the nucleotide termination mixes.

Add 8.0ul lightweight mineral oil to each of the four marked tubes containing the mastermix and nucleotide termination mixes.

Store on ice until ready to load into the thermocycler.

Parameters for cycle sequencing

1.	Denaturation	94°C	5 minutes	1X
2.	Denaturation	94°C	30 seconds	
	Annealing	60°C	30 seconds	35X
	Extension	72°C	60 seconds	
3.	Extension	72°C	5 minutes	1X
4.	Hold	6°C		

The temperature change during the cycles of the step 2 is desirably set to ramp at a rate of 1°C/sec.

At the end of the cycle sequencing reaction add 6.0ul of the Stop Loading Dye directly to each of the four tubes to stop the sequencing reaction. The sequencing samples are heated at 95°C for 2 minutes and then placed on ice before loading 2.0ul (from a total volume of 14ul) on the CLIPPER™ sequencer. The remainder of the sequencing reaction can be stored at -20°C for subsequent use.

The CLIPPER™ sequencer is set-up as described in the *OPENGENE Automated DNA Sequencing System User Manual*. Run parameters for the CLIPPER™ sequencer are 54°C/ 1300volts/ 0.5sec sampling/35min run/50% laser power. The samples loaded included 2 ul each of the forward and reverse sequencing reaction products for the target gene, differentially labeled, for example with CY5.0 and CY5.5 cyanine dye labels. Once the run is completed, the base-called data is analysed by comparison of the test sequence to the rpoB sequence database in GENELIBRARIAN™. This sequence alignment compares the test sequence to the standard control sequence and allows sequence ambiguities to be assessed. Once edited the test sequence can be screened for antibiotic resistance-associated mutations using GENELIBRARIAN™.

Testing for multiple types of antibiotic-resistance mutations can be carried out using a hierarchical assay, as summarized in Fig. 2. At present molecular biological methods for the detection of *M. tuberculosis* are only performed on AFB smear-positive sputum samples. These methods serve as confirmatory tests for the presence of *M. tuberculosis*. In addition to these molecular biological methods, the culture-based procedures for *M. tuberculosis* detection (BacTec liquid culture, agar plate and slant cultures) are performed in parallel. AFB smear-negative sputum samples are processed with only the culture-based detection procedures (Figure 1).

In the present invention both AFB smear-positive and smear-negative sputum samples can be processed using both culture-based and molecular biological methods. A limitation of the AFB stain methodology is its limit of detection. If a sputum sample has a mycobacterial concentration of less than 5000 bacteria/ul the AFB stain will be negative. In addition to this is the observation that the decontamination procedure used to prepare the sputum sample usually kills 10-20% of the mycobacteria present. This would suggest that two-thirds of the AFB smear-negative samples potentially contain mycobacteria. In practice 10-20% of the AFB smear-negative samples are culture-positive for *M. tuberculosis* (Ontario Public Health Laboratory). This level of mycobacteria is easily detected by molecular biological methods and is therefore incorporated in the present invention.

The hierarchy proposed incorporates tests that specifically detect *M. tuberculosis* (rpoB), detect mutations in genes associated with resistance to the "first-line" antibiotics used to treat *M. tuberculosis* infections (rpoB, katG, rpsL/s12, PR, embB, pncA)

and detect other species of mycobacteria (23S) in the absence of *M. tuberculosis* (Figure 2). Group I analyses are performed before both Group II and Group III. Group I analysis will provide information on the antibiotic resistance status to rifampin (*rpoB*), isoniazid (*katG*), streptomycin (*rpsL/s12*) and azithromycin (23S). In addition the *rpoB* amplification indicates the presence of *M. tuberculosis* and in the absence of *rpoB* amplification the 23S sequence allows identification of most of the clinically relevant mycobacterial species. Group II analysis provides information on antibiotic resistance mutations in the "second-line" antibiotics used to treat *M. tuberculosis* infections namely, isoniazid (PR), ethambutol (*embB*), pyrazinamide (*pncA*) and ciprofloxacin (*gyrA*). Group III contains gene targets in which mutations associated with antibiotic resistance are infrequently found. This protocol permits specific gene targets to be examined according to the local treatment procedures since the both antibiotics used to treat *M. tuberculosis* infections, and thus the associated antibiotic resistance mutation patterns, vary geographically. As shown in Figure 2 the culture-based methods are performed in parallel. The molecular biological methods would permit the identification of *M. tuberculosis* from both AFB smear-positive and smear-negative sputum samples and further provide information on the antibiotic resistance profile of these samples well in advance of current culture-based methods. This information would be crucial to the initiation of appropriate and effective antibiotic treatment regimens for *M. tuberculosis* infections.

Examples

A pool of DNA samples from antibiotic-sensitive *M. tuberculosis* isolates was obtained from the LCDC, Health and Welfare Canada, Ottawa, Ontario. Wild-type sequence traces, for all gene targets known to harbor mutations in antibiotic-resistant *M. tuberculosis*, were generated.

A panel of DNA samples from five phenotypic streptomycin-resistant *M. tuberculosis* isolates was obtained from the Public Health Laboratory, Ontario Ministry of Health, Toronto, Ontario. These DNA samples were examined for antibiotic resistance-associated mutations in all 10 antibiotic gene targets listed above. Streptomycin resistance-associated mutations were detected in the *rpsL/s12* gene in four isolates. Parallel antibiotic resistance-associated mutations in the *rpoB* (rifampin), *katG* (isoniazid), PR (isoniazid),

embB (ethambutol), pncA (pyrazinamide) and gyrA (ciprofloxacin) genes were also identified which underscores the importance of examining all the gene targets for first-line antibiotics used in the treatment of *M. tuberculosis*. A summary of the results is shown in Table 1.

The following references are cited herein and are incorporated herein by reference for all states which allow such incorporation.

- a DL Williams et al. (1994). Characterisation of rifampin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 38: 2380-2386.
- b WH Haas et al. (1997). Molecular analysis of katG gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *Antimicrob Agents Chemother* 41: 1601-1603.
- c S Sreevatsan et al. (1997). Analysis of the oxyR-ahpC region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. *Antimicrob Agents Chemother* 41: 600-606.
- d A Telenti et al. (1994). Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at the reference laboratory level. *Antimicrob Agents Chemother* 35: 719-723.
- e C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- f C Katsukawa et al. (1997). Characterisation of the rpsL and rrs genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J Appl Microbiol* 83: 634-640.
- g MA Lety et al. (1997). A single point mutation in the embB gene is responsible for resistance to ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 41: 2629-2633.
- h A Scorpio et al. (1997). Characterisation of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 41: 540-543.

i C Xu et al. (1996). Fluoroquinilone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *J Infect Disease* 174: 1127-1130.

j KA Nash et al. (1995). Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. *Antimicrob Agents Chemother* 39: 2625-2630.

Table 1

gene (antibiotic)	OPH#1 bp/codon/aa	OPH#2 bp/codon/aa	OPH#3 bp/codon/aa	OPH#4 bp/codon/aa	OPH#11 bp/codon/aa
rpoB (rifampin)	cac526tac, His526Tyr	tcg553llg, Ser553Leu	cac526gac, His526Asp	tcg553llg, Ser553Leu	wt
katG.1 (isoniazid)	agc513acc, Ser513Thr	agc513acc, Ser513Thr	agc513acc, Ser513Thr	wt	wt
oxyR-ahpC PR (isoniazid)	g541a	wt	wt	wt	g541a
fabG (isoniazid)	wt	wt	wt	wt	wt
rpsL/s12 (streptomycin)	wt	uag43agg, Lys43Arg	aag43agg, Lys43Arg	aag88agg, Lys88Arg	uag43agg, Lys43Arg
16s/rrs (streptomycin)	wt	wt	wt	wt	wt
embB (ethambutol)	wt	glc292llc, val292phe	wt	wt	wt
pncA (pyrazinamide)	tcg65lcl, Ser65Ser	wt	atl133aal, Ile133Asn	wt	tcg65lcl, Ser65Ser
gyrA (ciprofloxacin)	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr	agc95acc, Ser95Thr
23s (azithromycin)	wt	wt	wt	wt	wt

CLAIMS

1. A method for detection and characterization of *Mycobacterium tuberculosis* present in a sample, comprising the steps of:
 - (a) obtaining a sputum sample suspected of containing *M. tuberculosis*,
 - (b) performing a first sequencing procedure, with or without prior amplification, on the sample to detect the presence of *M. tuberculosis*, and if present to evaluate the *rpoB*, *katG*, *rpsL/s12* and *23S* genes for the presence of antibiotic-resistance inducing mutations; and
 - (c) if *M. tuberculosis* is detected in step (b), performing a second sequencing procedure, with or without prior amplification, on the sample to evaluate the additional genes for the presence of antibiotic-resistance inducing mutations.
2. The method of claim 1, wherein the second sequencing procedure evaluates *PR*, *embB*, *pncA* and *gyrA* genes for the presence of antibiotic-resistance mutations.
3. The method of claim 3, further comprising the step of performing a third sequencing procedure when *M. tuberculosis* was detected in step (b) to evaluate *16S/rrs* and *mabA* genes for the presence of antibiotic-resistance mutations.
4. The method of any of claims 1 to 3, wherein the first sequencing procedure for *rpoB* is performed using amplification primers as set forth in Seq. ID Nos. 1 and 2 and sequencing primers as set forth in Seq. ID. Nos. 3 and 4.
5. The method of any of claims 1 to 4, wherein the first sequencing procedure for *katG* is performed using amplification primers as set forth in Seq. ID Nos. 6 and 7 and sequencing primers as set forth in Seq. ID. Nos. 8 and 9.
6. The method of any of claims 1 to 5, wherein the first sequencing procedure for *rpsL/s12* is performed using amplification primers as set forth in Seq. ID Nos. 21 and 22 and sequencing primers as set forth in Seq. ID. Nos. 23 and 24.

7. The method of any of claims 1 to 6, wherein the second sequencing procedure for 23S is performed using amplification primers as set forth in Seq. ID Nos. 46 and 47 and sequencing primers as set forth in Seq. ID. Nos. 48 and 49.
8. The method of any of claims 1 to 7, wherein the second sequencing procedure for PR is performed using amplification primers as set forth in Seq. ID Nos. 11 and 12 and sequencing primers as set forth in Seq. ID. Nos. 13 and 14.
9. The method of any of claims 1 to 8, wherein the second sequencing procedure for pncA is performed using amplification primers as set forth in Seq. ID Nos. 36 and 37 and sequencing primers as set forth in Seq. ID. Nos. 38 and 39.
10. The method of any of claims 1 to 9, wherein the second sequencing procedure for embB is performed using amplification primers as set forth in Seq. ID Nos. 31 and 32 and sequencing primers as set forth in Seq. ID. Nos. 33 and 34.
11. The method of any of claims 1 to 10, wherein the second sequencing procedure for gyrA is performed using amplification primers as set forth in Seq. ID Nos. 41 and 42 and sequencing primers as set forth in Seq. ID. Nos. 43 and 44.
12. The method of any of claims 2 to 11, wherein the third sequencing procedure for 16S/rrs is performed using amplification primers as set forth in Seq. ID Nos. 26 and 27 and sequencing primers as set forth in Seq. ID. Nos. 28 and 29.
13. The method of any of claims 2 to 12, wherein the third sequencing procedure for mabA is performed using amplification primers as set forth in Seq. ID Nos. 16 and 17 and sequencing primers as set forth in Seq. ID. Nos. 18 and 19.
14. A kit for evaluation of antibiotic-resistance mutations in a sample of *Mycobacterium tuberculosis*, comprising one or more pairs of amplification primers and one or more matched pairs of sequencing primers for amplification and sequencing regions within

the genome of *M. tuberculosis*, characterized in that the amplification and sequencing primer pairs are selected from among:

- (a) amplification primers of Seq. ID Nos. 1 and 2 in combination and sequencing primers of Seq. ID Nos. 3 and 4;
- (b) amplification primers of Seq. ID Nos. 6 and 7 in combination and sequencing primers of Seq. ID Nos. 8 and 9;
- (c) amplification primers of Seq. ID Nos. 11 and 12 in combination and sequencing primers of Seq. ID Nos. 13 and 14;
- (d) amplification primers of Seq. ID Nos. 16 and 17 in combination and sequencing primers of Seq. ID Nos. 18 and 19;
- (e) amplification primers of Seq. ID Nos. 21 and 22 in combination and sequencing primers of Seq. ID Nos. 23 and 24;
- (f) amplification primers of Seq. ID Nos. 26 and 27 in combination and sequencing primers of Seq. ID Nos. 28 and 29;
- (g) amplification primers of Seq. ID Nos. 31 and 32 in combination and sequencing primers of Seq. ID Nos. 33 and 34;
- (h) amplification primers of Seq. ID Nos. 36 and 37 in combination and sequencing primers of Seq. ID Nos. 38 and 39;
- (i) amplification primers of Seq. ID Nos. 41 and 42 in combination and sequencing primers of Seq. ID Nos. 43 and 44; and
- (j) amplification primers of Seq. ID Nos. 46 and 47 in combination and sequencing primers of Seq. ID Nos. 48 and 49.

1/2

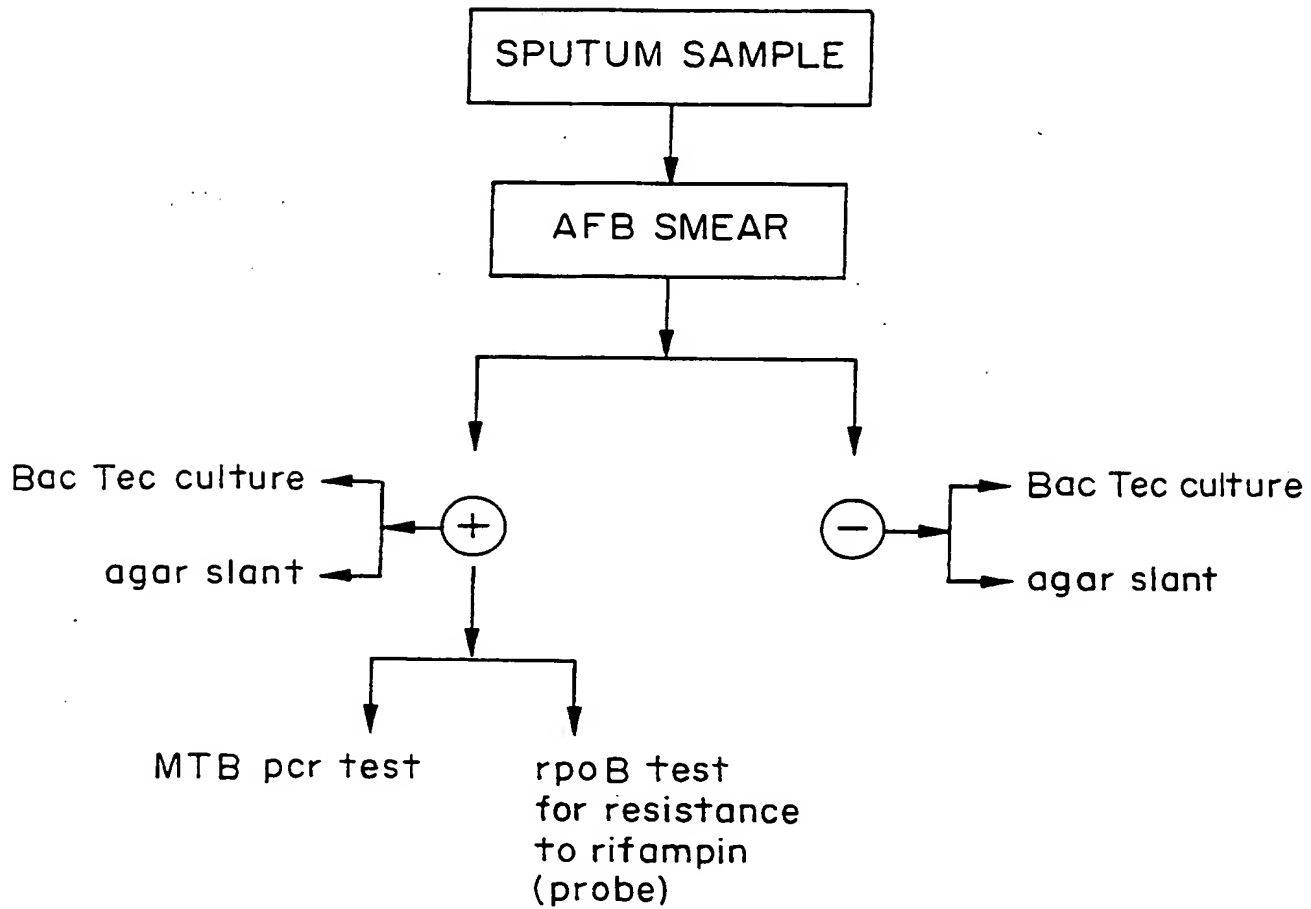


FIG. 1

2/2

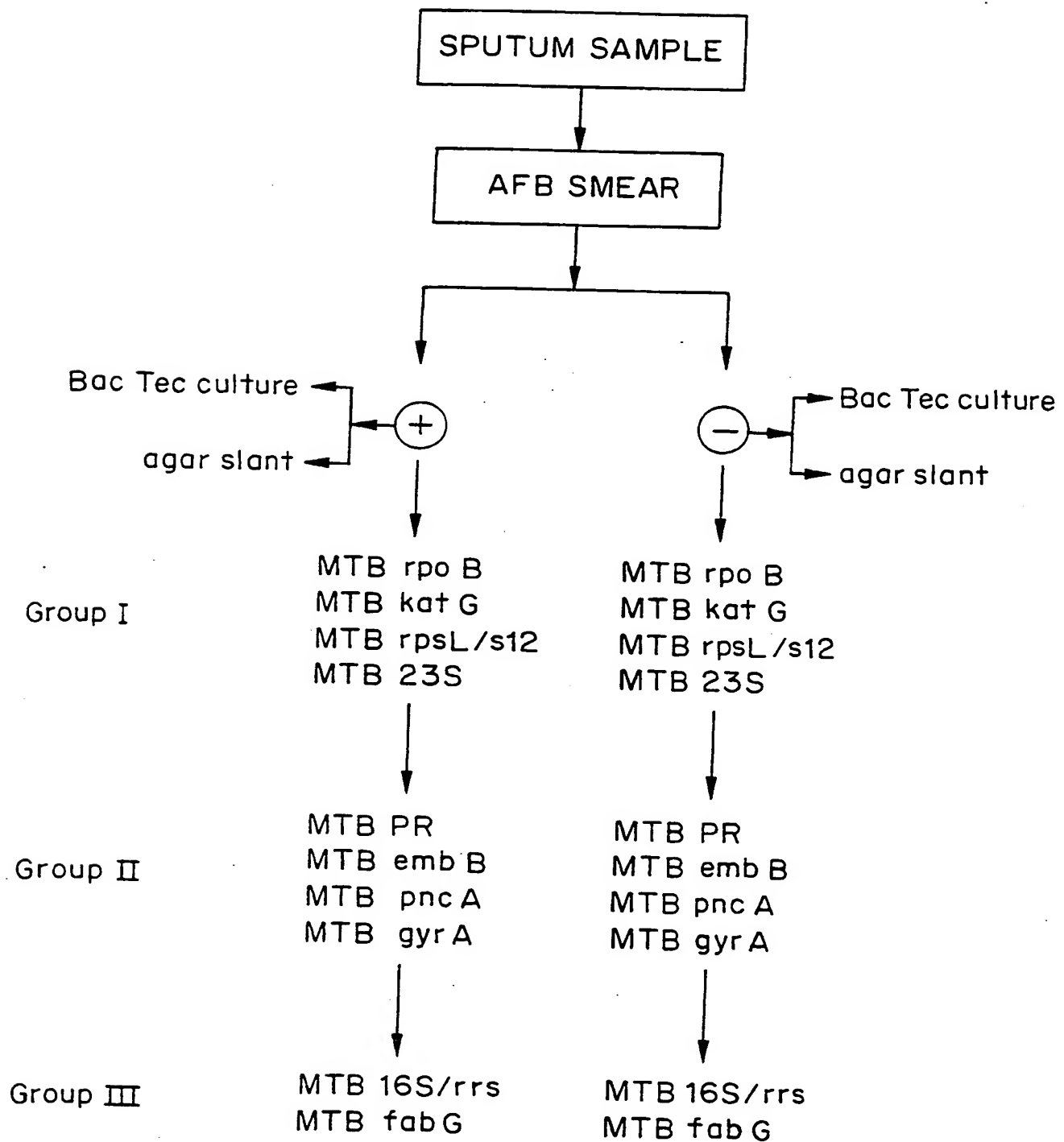


FIG. 2

1/15

SEQUENCE LISTING

<110> Visible Genetics Inc.
Shipman, Robert

<120> Method and Kit for the Characterization of
Antibiotic-Resistance Mutations in Mycobacterium
tuberculosis

<130> VGEN.P-055-WO

<140>

<141>

<150> 60/111,794

<151> 1998-12-11

<160> 50

<170> PatentIn Ver. 2.1

<210> 1

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-F amplification primer

<400> 1

tacggtcggc gagctgatcc

20

<210> 2

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-R amplification primer

<400> 2

tacggcggtt cgatgaaccc

20

<210> 3

<211> 20

<212> DNA

2/15

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-5s sequencing primer

<400> 3

tacggtcggc gagctgatcc

20

<210> 4

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB-3s sequencing primer

<400> 4

tacggcgctt cgatgaaccc

20

<210> 5

<211> 480

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> rpoB (rifampin resistance)

<400> 5

```

aaaccgacga catcgaccac ttcggcaacc gccgcctgcg tacggtcggc gagctgatcc 60
aaaaccagat ccgggtcggc atgtcgcgga tggagcgggt ggtccgggag cggatgacca 120
cccaggacgt ggaggcgatc acaccgcaga cgttgatcaa catccggccg gtggtcgccg 180
cgatcaagga gttcttcggc accagccagc tgagccaatt catggaccag aacaacccgc 240
tgtcgggggt gaccacaag cgcgcactgt cggcgctggg gcccggcggt ctgtcacgtg 300
agcgtgccgg gctggaggtc cgcgacgtgc acccgtcgca ctacggccgg atgtgcccga 360
tcgaaacccc tgagggggccc aacatcggtc tgatcggtc gctgtcgggt tacgcgcggg 420
tcaacccggt cgggttcata gaaacgccgt accgcaaggt ggtcgacggc gtggttagcg 480

```

<210> 6

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> katG-F amplification primer

<400> 6
atggggctga tctacgtgaa 20

<210> 7
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-R amplification primer

<400> 7
ggtgttccag ccagcgacgc 20

<210> 8
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-5s sequencing primer

<400> 8
atggggctga tctacgtgaa 20

<210> 9
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG-3s sequencing primer

<400> 9
ggtgttccag ccagcgacgc 20

<210> 10
<211> 660
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> katG (isoniazid resistance)

4/15

<400> 10

```

gctcggcgat gagcggtaca gcggttaagcg ggatctggag aaccgcgtgg ccgcgggtgca 60
gatggggctg atctacgtga acccgaggag gccgaacggc aaccgggacc ccatggccgc 120
ggcggtcgac attcgcgaga cgtttcggcg catggccatg aacgacgtcg aaacagcggc 180
gctgatcgtc ggcggtcaca ctttcggtaa gacctatggc gccggcccg ccgatctggt 240
cggccccgaa cccgaggctg ctccgctgga gcagatgggc ttgggctgga agagctcgta 300
tggcaccgga accggttaag acgcgatcac cagcggcatc gaggtcgat ggacgaacac 360
cccgacgaaa tgggacaaca gtttcctcga gatcctgtac ggctacgagt gggagctgac 420
gaagagccct gctggcgctt ggcaatacac cgccaaggac ggcgcgggtg ccggcaccat 480
cccgaccg ttcggcgggc cagggcgctc cccgacgat ctggccactg acctctcgt 540
gcgggtggat ccgatctatg agcggatcac gcgtcgctgg ctggaacacc ccgaggaatt 600
ggccgacgag ttcgccaagg cctgggtacaa gctgatccac cgagacatgg gtcccgttgc 660

```

<210> 11

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> PR-F amplification primer

<400> 11

accactgctt tgccgccacc

20

<210> 12

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> PR-R amplification primer

<400> 12

ccgatgagag cggtgagctg

20

<210> 13

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> PR-5s sequencing primer

<400> 13

accactgctt tgccgccacc

20

5/15

<210> 14
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> PR-3s sequencing primer

<400> 14
ccgatgagag cggtgagctg

20

<210> 15
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> oxyR-ahpC intergenic region (PR)

<400> 15
atgccctggg ggtgcaccga gaccggcttc cgaccaccgc tcgccgcaac gtcgactggc 60
tcataatcgag aatgcttgcg gcactgctga accactgctt tgccgccacc gcggcgaacg 120
cgcgaagccc ggccacggcc ggctagcacc tcttggcggc gatgccgata aatatggtgt 180
gatatatcac ctttgccctga cagcgacttc acggcacgat ggaatgtcgc aaccaaatgc 240
attgtccgct ttgatgatga ggagagtcac gccactgcta accattggcg atcaattccc 300
cgctaccag ctcaccgctc tcacggcgcg tgacctgtcc aagggtcgacg ccaagcagcc 360
cggcgactac ttcaccacta tcaccagtga cgaacaccca ggcaagtggc ggggtggtgtt 420

<210> 16
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> fabG-F amplification primer

<400> 16
cctcgctgcc cagaaaggga

20

<210> 17
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

6/15

<220>

<223> fabG-R amplification primer

<400> 17

atcccccggt ttccctccggt

20

<210> 18

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-5s sequencing primer

<400> 18

cctcgctgcc cagaaaggga

20

<210> 19

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG-3s sequencing primer

<400> 19

atcccccggt ttccctccggt

20

<210> 20

<211> 360

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> fabG (isoniazid resistance)

<400> 20

agcgcgacat acctgctgcg caattcgtag ggcgtcaata caccgcgagc cagggcctcg 60
 ctgcccagaa agggatccgt catggtcgaa gtgtgctgag tcacaccgac aaacgtcacg 120
 agcgtaaccc cagtgcgaaa gttcccgcg gaaatcgag ccacgttacg ctctgggaca 180
 taccgatttc ggcccggccg cggcgagacg ataggttgc ggggtgactg ccacagccac 240
 tgaaggggcc aaacccccat tegtatcccg ttcagtcctg gttaccggag gaaaccgggg 300
 gatcgggctg gcgatcgac acgggctggc tgccgacggc cacaagggtg ccgtcaccca 360

7/15

<210> 21
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-F amplification primer

<400> 21
cggtagatgc caaccatcca 20

<210> 22
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-R amplification primer

<400> 22
gcatcagccc ttctccttct 20

<210> 23
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-5s sequencing primer

<400> 23
cggtagatgc caaccatcca 20

<210> 24
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> s12-3s sequencing primer

<400> 24
gcatcagccc ttctccttct 20

8/15

<210> 25
<211> 420
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> rpsL/s12 (streptomycin resistance)

<400> 25
cggtagatgc caaccatcca gcagctgggc cgcaagggtc gtcgggacaa gatcagtaag 60
gtcaagaccg cggctctgaa gggcagcccg cagcgtcgtg gtgtatgcac ccgctgttac 120
accaccactc cgaagaagcc gaactcggcg cttcggaagg ttgcccgcggt gaagttgacg 180
agtcaggtcg aggtcacggc gtacattccc ggcgagggcc acaacctgca ggagcactcg 240
atggtgctgg tgcgcggcgg ccgggtgaag gacctgctg gtgtgcgcta caagatcatc 300
cgcggttcgc tggatacgca ggggtgtcaag aaccgcaaac aggcacgcag ccgttacggc 360
gctaagaagg agaagggtg atgccacgca aggggcccgc gcccaagcgt ccgttggtca 420

<210> 26
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-F amplification primer

<400> 26
ggtgatctgc cctgcacttc g

21

<210> 27
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> 16S-R amplification primer

<400> 27
cgtcacccca ccaacaagct g

21

<210> 28
<211> 21
<212> DNA
<213> Mycobacterium tuberculosis

9/15

<220>

<223> 16S-5s sequencing primer

<400> 28

ggtgatctgc cctgcacttc g

21

<210> 29

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S-3s sequencing primer

<400> 29

cgtcacccca ccaacaagct g

21

<210> 30

<211> 147

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 16S/rrs (streptomycin resistance)

<400> 30

cgtgggtgat ctgccctgca cttcgggata agcctgggaa actgggtcta ataccggata 60
ggaccacggg atgcatgtct tgtggtggaa agcgctttag cggtgtggga tgagcccgcg 120
gcctatcagc ttgttggtgg ggtgacg 147

<210> 31

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-F amplification primer

<400> 31

cggcaagctg gcgcaccttc a

21

<210> 32

<211> 21

<212> DNA

10/15

<213> Mycobacterium tuberculosis

<220>

<223> embB-R amplification primer

<400> 32

agccagcaca ctagcccggc g

21

<210> 33

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-5s sequencing primer

<400> 33

cggcaagctg gcgcaccttc a

21

<210> 34

<211> 21

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB-3s sequencing primer

<400> 34

agccagcaca ctagcccggc g

21

<210> 35

<211> 300

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> embB (ethambutol resistance)

<400> 35

cggcatgcgc cggctgattc cggcaagctg gcgcaccttc accctgaccg acgccgtggc 60
 gata-tcggc ttcctgctct ggcattgcat cggcgcgaat tcgtcggacg acggctacat 120
 cctgggcatg gcccgagtcg ccgaccacgc cggctacatg tccaactatt tccgctgggt 180
 cggcagccccg gaggatccct tcggctggta ttacaacctg ctggcgctga tgacccatgt 240
 cagcgacgcc agtctgtgga tgcgcctgcc agacctggcc gccgggctag tgtgctggct 300

11/15

<210> 36
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 36
atgcgggctg tgatcatcgt

20

<210> 37
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-F amplification primer

<400> 37
tcaggagctg caaaccaact

20

<210> 38
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-5s sequencing primer

<400> 38
atgcgggctg tgatcatcgt

20

<210> 39
<211> 20
<212> DNA
<213> Mycobacterium tuberculosis

<220>
<223> pncA-3s sequencing primer

<400> 39
tcaggagctg caaaccaact

20

12/15

<210> 40
 <211> 561
 <212> DNA
 <213> Mycobacterium tuberculosis
 <220>
 <223> pncA (pyrazinamide resistance)

<400> 40
 atgCGggcgt tgatcatcgt cgacgtgcag aacgacttct gCGaggggtgg ctCGctggcg 60
 gtaaccggtg gcgcgcgcgt ggcccgcgcc atcagcgact acctggccga agcggcggac 120
 taccatcacg tcgtggcaac caaggacttc cacatcgacc cgggtgacca cttctccggc 180
 acaccggact attcctcgtc gtggccaccg cattgcgtca gcggtactcc cggcgcggac 240
 ttccatccca gtctggacac gtcggcaatc gaggcgggtg tctacaaggg tgcctacacc 300
 ggagcgtaca gcggcttcga aggagtcgac gagaacggca cgcactgct gaattggctg 360
 cggcaacgcg gcgtcgatga ggtcgatgtg gtcggtattg ccaccgatca ttgtgtgcgc 420
 cagacggccg aggacgcggt acgcaatggc ttggccacca gggtgctggg ggacctgaca 480
 gcgggtgtgt cggccgatac caccgtcgcc gcgctggagg agatgcgcac cgccagcgtc 540
 gagttggttt gcagctcctg a 561

<210> 41
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-F amplification primer

<400> 41
 cagctacatc gactatgcga

20

<210> 42
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-R amplification primer

<400> 42
 gggcttcggt gtacctcatc

20

<210> 43
 <211> 20

13/15

<212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-5s sequencing primer

<400> 43
 cagctacatc gactatgcga

20

<210> 44
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA-3s sequencing primer

<400> 44
 gggcttcggt gtacctcatc

20

<210> 45
 <211> 420
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> gyrA (fluoroquinilone/ciprofloxacin resistance)

<400> 45
 cgaccggatc gaaccggttg acatcgagca ggagatgcag cgcagctaca tcgactatgc 60
 gatgagcgtg atcgtcggcc gcgcgctgcc ggaggtgcgc gacgggctca agcccgtgca 120
 tcgccgggtg ctctatgcaa tgttcgattc cggcttcgcg ccggaccgca gccacgcca 180
 gtcggcccg tgggttgccg agaccatggg caactaccac ccgcacggcg acgcgtcgat 240
 ctacgacagc ctgggtgcga tggcccagcc ctggtcgcgt cgctaccgc tgggtggacgg 300
 ccagggcaac ttcggctcgc caggcaatga cccaccggcg gcgatgaggt acaccgaagc 360
 ccggctgacc ccgttggcga tggagatgct gagggaaatc gacgaggaga cagtcgattt 420

<210> 46
 <211> 20
 <212> DNA
 <213> Mycobacterium tuberculosis

<220>
 <223> 23S-F amplification primer

14/15

<400> 46

cgaaattcct tgcgggtaa

20

<210> 47

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-R amplification primer

<400> 47

gtatttcaac aacgactcca

20

<210> 48

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-5s sequencing primer

<400> 48

cgaaattcct tgcgggtaa

20

<210> 49

<211> 20

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S-3s sequencing primer

<400> 49

gtatttcaac aacgactcca

20

<210> 50

<211> 300

<212> DNA

<213> Mycobacterium tuberculosis

<220>

<223> 23S (macrolide/azithromycin resistance)

15/15

<400> 50

```
gccccagtaa acggcggtgg taactataac catcctaagg tagcgaaatt ccttgtcggg 60
taagttccga cctgcacgaa tggcgtaacg acttcccaac tgtctcaacc atagactcgg 120
cgaaattgca ctacgagtaa agatgctcgt tacgcgcggc aggacgaaaa gaccccgga 180
ccttcactac aacttggtat tgggtgttcgg tacggtttgt gtaggatagg tgggagactt 240
tgaagcacag acgccagttt gtgtggagtc gttgttgaaa taccactctg atcgtattgg 300
```

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/CA 99/01177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPUR V ET AL: "Application of automated DNA sequence analysis for mycobacterium species identification and detection of mutations associated with antibiotic resistance in Mycobacterium tuberculosis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 34, 1994, page 163 XP000901974 see abstract 071 ---	1-3
X	SUZUKI Y ET AL: "Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16SrRNA gene" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 5, May 1998 (1998-05), pages 1220-5, XP000901934 the whole document ----- -/-	1-3

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

13 April 2000

Date of mailing of the international search report

28/04/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	HONORE N ET AL: "Streptomycin resistance in mycobacteria" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 38, no. 2, February 1994 (1994-02), pages 238-42, XP000901931 page 239, paragraph 2 ---	1-3
Y	SCORPIO A ET AL: "Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 41, no. 3, March 1997 (1997-03), pages 540-543, XP000901990 page 540 -page 542, paragraph 4 ---	1-3
Y	ALANGADEN GJ ET AL: "Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 42, no. 5, May 1998 (1998-05), pages 1295-97, XP000901991 the whole document ---	1-3
Y	HEYM B ET AL: "IMPLICATIONS OF MULTIDRUG RESISTANCE FOR THE FUTURE OF SHORT-COURSE CHEMOTHERAPY OF TUBERCULOSIS: A MOLECULAR STUDY" LANCET THE,GB,LANCET LIMITED. LONDON, vol. 344, no. 8918, 30 July 1994 (1994-07-30), pages 293-298, XP002039609 ISSN: 0140-6736 the whole document ---	1-3
Y	WO 97 23650 A (DUNN JAMES M ;LEUSHNER JAMES (CA); STEVENS JOHN K (CA); VISIBLE GE) 3 July 1997 (1997-07-03) page 10, paragraph 1 -page 11, paragraph 4; example 7 ---	1-3
A	WO 95 33074 A (MAYO FOUNDATION ;HOFFMANN LA ROCHE (US)) 7 December 1995 (1995-12-07) page 3, paragraph 3 -page 6, paragraph 3 ---	1-3
A	WO 95 33851 A (INNOGENETICS NV ;BEENHOUWER HANS DE (BE); PORTAELS FRANCOISE (BE);) 14 December 1995 (1995-12-14) claims 2,22 ---	1-3
	-/--	

INTERNATIONAL SEARCH REPORT

Int. .tional Application No

PCT/CA 99/01177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P, X	<p>NUESCA D ET AL: "RAPID DETECTION OF ANTIBIOTIC RESISTANCE-ASSOCIATED MUTATIONS IN 10 GENE TARGETS IN MYCOBACTERIUM TUBERCULOSIS USING THE OPENGENE(R) SYSTEM"</p> <p>ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 30 May 1999 (1999-05-30) - 3 June 1999 (1999-06-03), page 636 XP000891874 see abstract U-13</p> <p style="text-align: center;">-----</p>	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/01177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723650 A	03-07-1997	US 5834189 A	10-11-1998
		AU 1426297 A	17-07-1997
		CA 2239896 A	03-07-1997
		EP 0870059 A	14-10-1998
		WO 9741257 A	06-11-1997
		US 5888736 A	30-03-1999
		US 5981186 A	09-11-1999
		US 6007983 A	28-12-1999
WO 9533074 A	07-12-1995	US 5643723 A	01-07-1997
		AU 2605795 A	21-12-1995
WO 9533851 A	14-12-1995	AU 703947 B	01-04-1999
		AU 2789695 A	04-01-1996
		BR 9507960 A	02-09-1997
		CZ 9603612 A	16-07-1997
		EP 0771360 A	07-05-1997
		JP 10500857 T	27-01-1998

THIS PAGE BLANK (USPTO)